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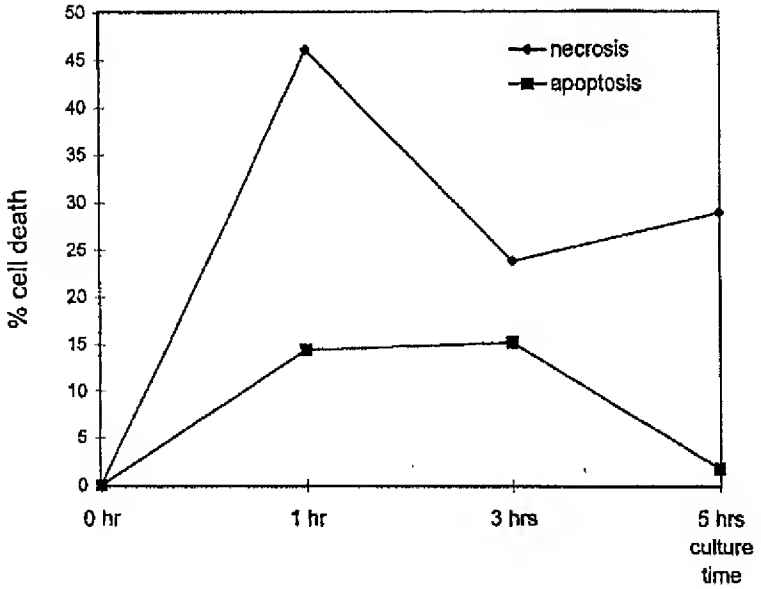

(57) Abstract

The present invention relates to the use as an active substance of molecules binding to β_1-integrins and with the capacity to kill cells having β_1-integrins for production of pharmaceuticals for treatment of conditions dependent on cells having β_1-integrins such as cancer and conditions dependent on T lymphocytes and fibrotic conditions. Especially the invention relates to the use of Invasin (Inv) from Yersinia (Yer) species, subfragments, variants, and peptides thereof, microorganisms producing invasin and invasin like substances and antibodies binding β_1-integrins and with the capacity to kill cells having β_1-integrins.

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New use.

The present invention relates to the use as an active substance of molecules binding to β_1-integrins and with the capacity to kill cells having β_1-integrins for production of pharmaceuticals for treatment of conditions dependent on cells having β_1-integrins such as cancer and conditions dependent on T lymphocytes and fibrotic conditions.

Especially the invention relates to the use of Invasin (Inv) from Yersinia (Yer) species, subfragments, variants, and peptides thereof, microorganisms producing invasin and invasin like substances and antibodies binding β_1-integrins and with the capacity to kill cells having β_1-integrins.

The conditions to be treated according to the invention are e.g. cancers, conditions dependent on or caused by T lymphocytes, e.g. autoimmune diseases, such as rheumatoid arthritis, and rejection of transplants and fibrotic conditions.

The invention also relates to the use of the corresponding genes for production of pharmaceuticals for gene therapy, pharmaceutical compositions and a method for treatment of conditions mentioned herein.

Yer species are responsible for a broad range of diseases in humans ranging from acute bowel disease to extraintestinal manifestations such as reactive arthritis, erythema nodosum and uveitis (Mandel, G. et al. Principles and practice of Infectious diseases, 2nd Ed. Churchill Livingstone, New York, 1988). Yer binds to β_1-integrins and thereby invade host cells (Brett, S. T. et al. Eur. J. Immunol. 23, 1608-1614, 1993). The Inv protein of Y. pseudotuberculosis and Y. enterocolitica accounts for this interaction and binds to multiple β_1-integrins via the C-terminal 192 amino acids (Leong, J.M.,EMBO J. 9, 1979-1989, 1990). Inv also provides costimulatory activity for T cells in vitro by binding to $\alpha_4\beta_1$ integrin which is involved in binding to VCAM on endothelial cells and to fibronectin (Isberg, R., Trends Microbiol. 2, 10-14, 1994) . Inv protein is also a potent inducer of pseudopodia formation and chemotactic and haptotactic migration in T cells

(Arencibia, I., J. Immunol. 159, 1853-1859).

It has now turned ot that Yer can kill T cells and several non-lymphoid tumour cells. This killing can be attributed to the action of a molecule of Yer species, invasin (Inv). Inv is a molecule of 103 kD the amino acid sequence of which is known. The killing effect induced by Yer Inv is mediated via bounding to β_1 integrins. Thus, Inv protein causes necrotic-apoptotic cell death in T lymphocytes while an Inv preparation from the same strain carrying an insertion in the binding site is inactive. Killing by Inv does not require live bacteria.

EP 0 211 543 describe a vaccine that may contain the gene coding for Yersinia inv. It has however not been disclosed before that invasin and microorganisms carrying this protein can kill cells having β_1-integrin such as T lymphocytes and tumour cells.

The present invention relates to the use of Invasin, subfragments and variants thereof, and micro-organisms producing such substances, antibodies with invasin like effect, that bind to β_1-integrins and kill cells having β_1-integrin for production of pharmaceuticals for treatment of conditions dependent on cells having β_1-integrin such as cancer, autoimmune conditions dependent on T-lymphocytes, and conditions dependent on fibroblasts such as fibrotic conditions.

Any variant, modification, subfragment, or peptide of the Invasin protein, antibody or Invasin like molecule that binds to β_1-integrin and kills cells having β_1-integrins may be used according to the invention. The invention comprises any invasin or invasin like molecule produced by solubilizing of the molecule from any microorganism containing or bearing such a molecule, such as those mentioned below. The molecule can also be synthesized or produced by recombinant technology. Also fusion proteins comprising one or more invasin molecules or one or more variants or fragments thereof are within the frame of the invention.

Thus, by invasin like molecule we understand any molecule and fragment thereof that binds to β_1-integrins and kills cells having β_1-integrin.

Variants comprise any addition, deletion, substitution or addition of amino acids in the protein. Such variants are within the scope of the invention as long as they are capable binding to cells having β_1-integrins and kill them. Preferable invasin is used. The amino acid sequence of invasin may be found in reference Isberg, R. R., et al. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells, Cell 1987 Aug 28; 50(5): 769-78. Any part of invasin or any variant of invasin or any variant of a fragment thereof binding to β_1-integrins and with the capacity to kill cells having β_1-integrins are encompassed by the invention.

It would also be possible to use such antibodies that bind to β_1-integrins or associated proteins and kill cells having β_1-integrins. Methods for producing such antibodies are well known to the skilled person and are described e.g. in the Current Protocols of Immunology, Vol. I, Eds. Coligan, A. M. et al. John Wley and Sons, Inc., New York and Bodey, B. et al. Anticancer Res. 16(2):661-674, 1996. Killing of cells can be determined as described in example 2 using the Annexin test.

Monoclonal antibodies to β_1-integrins with the capacity to kill cells having β_1-integrins are produced by standard hybridoma technology using purified β_1-integrin as immunogen. The screening procedure consists of testing supernatants containing antibodies with killing activity and binding β_1-integrin on lymphoid cells. Different screening procedures are possible for identification of clones producing antibodies with these characteristics. Methods for producing such antibodies are well known to the skill person and are described e.g. in the Current Protocols of Immunology, Vol. I, Eds. Coligan, A. M. et al. John Weley and Sons, Inc, New York 1994 and Bodey, B. et al. Anticancer Res. 16(2):661-674, 1996. Killing of cells can be determined as described in example 2 using the Annexin test (see below).

It is also possible to use whole or part of such micro-organisms that produce the invasin or invasin like molecule. The micro-organisms may be live, attenuated or dead, e.g. in fixed form. Extracts from such micro-organisms or nutrient media containing the active invasin like molecules may be used.

Suitable micro-organisms are those which contain or produce active invasin or invasin like molecules or other proteins with the capacity to bind to β_1-integrins and kill tumour cells and/or T-lymphocytes such as Yersinia pseudotuberculosis, Yersinia pestis, Yersinia enterocolitica, Salmonella, Meningococci, Shigella, etc. Binding of Inv to cells can be detected by means of antibodies as described in example 5 below.

Invasin may be produced from any Yer species e.g. Yer pseudotuberculosis strainYPIII(pIB1)(Inv positive)(Bölin, I., et al. Infect. Immun. 37, 506-512, 1982) as described in Example 1. It can also be produced by hybrid technology or synthetically from the corresponding gene Falkow et al., reviews of Infectious Diseases, 9 Supp. 5 S459-S455 (1987) .

According to the invention the invasin or invasin like molecule that have the above mentioned effects or fixed bacteria such as Yersinia pseudotuberculosis, Yersinia pestis or Yersinia enterocolitica, or an extract of live or fixed bacteria may be coupled to a target-seeking antibody preferably a monoclonal antibody, Fab or Fc portion of a antibody or a hybrid antibody or other protein.

The targeting molecule is a molecule that preferentially seeks the β_1-integrin cells to be killed i.e. the target cancer cells or the target T-lymphocytes. Monoclonal or polyclonal antibodies or parts of antibodies directed to the target cells may be produced by conventional methods known in the art such as those mentioned above. The targeting molecule should bind to some other site than the β_1-integrins in order not to interfere with the effect of the invasin like molecule.

The invasin like molecules or micro-organisms producing such molecules with the effect according to the invention can be used for treatment of conditions that depend on T-lymphocytes. Such conditions are those where β_1-integrins on T-lymphocytes bind to mammalian cells especially to endothelial cells or to the extracellular matrix such as autoimmune diseases e.g. rheumatoid arthritis and fibrotic conditions e.g. pulmonary fibrosis.

The invasin-like molecules or micro-organisms producing such molecule with the effect according to the invention can also be used for treatment of cancer cells having β_1-integrins.

By β_1-integrins we understand the whole β_1-integrin family of proteins (see references Hynes, R., Integrins: versality, modulation, and signaling in cell adhesion. Cell, 69, 11, 1992 and Hemler, M. E., VLA proteins in the integrin family: structures, functions, and their role on leukocytes, Annu. Rev. Immunol., 8, 365, 1990).

The composition for use according to the invention may be prepared for any route of administration, e.g. oral, rectal, intravenous, cutaneous or subcutaneous, nasal, intratecal, intramuscular or intraperitoneal. The precise nature of the carrier or other material will depend on the route of administration. For a parenteral administration, a parenterally acceptable aqueous solution is employed, which is pyrogen free and has requisite pH, isotonicity and stability. Those skilled in the art are well able to prepare suitable solutions and numerous methods are described in the literature. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be included, as required. Dosage levels can be determined by those skilled in the art, taking into account the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16 th edition Osol, a. (ed) 1980.

Genes coding for the invasin or invasin like molecule may be used according to invention for gene therapy. The invasin gene or sequence of the invasin gene is then used as part of specially designed gene therapy for delivery in specific locations as brain, joints, liver, etc, as well as in other therapeutic method(s).

For a review of gene therapy procedures, see Anderson, Science (1992) 256:808-813; Nabel and Felgner (1993) TIBTECH 11:211-217; Haddada et al. (1995) in Current Topics in Microbiology and Immunology, Doerfler and Böhm (eds) Springer-Verlag, Heidelberg, Germany, and Yu et al. Gene Therapy (1994) 1:13-26.

Delivery of the genetic material into the cell is the first critical step in gene therapy treatment of disease. A large number of delivery methods are well known to those of skill of the art. Such methods include, for example, liposome-based gene delivery (Debs and Zhu, 1993) WO 93/24640; Mannino and Gould-Fogerite (1988)BioTechniques 6(7):682-691; Rose U. S. Pat No 5,279,833; replication -defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome (see, e.g., Miller et al., 1990, Mol. Cell Biol. 10:4239; and Cornetta et al. Hum. Gene Ther. 2:215, 1991.) Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian immuno deficiency virus (SIV), human immuno deficiency virus (HIV) and combinations thereof. See, e.g., Buchscher et al. (1992) J. Virol. 66(5):2731-2739 and Miller et al. J. Virol. 65:2220-2224 (1991).

AAV-based vectors are also use to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and in the in vivo and ex vivo gene therapy procedures. See, West et al. (1987) Virology 160:38-47; Carter et al. (1989) U. S. Patent No. 4,797,368; Carter et al. WO 93/24642 (1993) and Muzyczka (1994) J. Clin. Invest. 94:1351 for an overview of AAV vectors. Construction of recombinants AAVvectors are described in a number of publications, including Lebkowski, U. S. Patent No. 5,173,414 and Samulski et al. (1989) J. Virol. 63:3822-3828. Cell lines that can be transformed bu rAAV include those described in Ledkowski et al. (1988) Mol.

Cell Biol. 8:3988-3996. (All cited publications herein are included by reference).

The invention also relates to a method for killing of cells having β_1-integrin, comprising exposing such cells to an effective amount of invasin or invasin like molecule, live or fixed Yersinia pseudotuberculosis, Yersinia pestis or Yersinia enterocolitica, an extract of live or fixed Yersinia pseudo/pestis/enterocolitica or to Yersinia pseudo/pestis/enterocolitica.

The invention also relates to a method for killing of T lymphocytes comprising exposing the lymphocytes to an effective amount of invasin or invasin like molecule, live or fixed Yersinia pseudotuberculosis, Yersinia pestis or Yersinia enterocolitica, an extract of live or fixed Yersinia pseudo/pestis/entero or to Yersinia pseudo/pestis/enterocolitica.

Further the invention concerns a method for killing of tumour cells comprising exposing the tumour cells to an effective amount of invasin or invasin like molecule or live or fixed Yersinia pseudotuberculosis, Yersinia pestis or Yersinia enterocolitica, an extract of live or fixed Yersinia pseudo/pestis/enterocolitica or to Yersinia pseudo/pestis/enterocolitica invasin.

The invention is illustrated by the following figures :

Fig. 1 and 2 illustrate development of necrotic and apoptotic cell death in T lymphocytes, Jurkat cells, exposed to fixed Yersinia pseudotuberculosis (1) and Yersinia enterocolitica (2).

Fig. 3) shows cell death caused by Y1 and Y2 (truncated) extracts from Yersinia pseudotuberculosis and by MBP-Inv fusion proteins Inv-740 and Inv-497 in T lymphocytes, Jurkat and AF-24 cell lines.

Fig. 4) shows comparison of the capacity of three different extracts from Y. pseudotuberculosis to induce killing of Molt-4 T lymphocytes. (Example 4)

Fig. 5) shows binding of Inv from Y1 from Yersinia pseudotuberculosis and Y2 (truncated) extracts to T lymphocytes, Jurkat and AF-24 cell lines.

Fig. 6) shows cell death-inducing effect on T lymphocytes, Jurkat cells, of an extract of Yersinia pseudotuberculosis (Y1E) before and after removal of Inv (100 kD).

Fig. 7). shows the influence of monoclonal antibodies to cell surface antigens on Inv-induced cell death in the presence of Yersinia enterocolitica Y1E and Y2E (truncated) in Jurkat cells.

Fig. 8) shows reduction of Inv-induced cell death in a β_1-integrin negative cell line (HAB.7) compared with its parental line (HPB-ALL). The cells were cultured with fixed Yersinia pseudotuberculosis for 16 hours before determination of cell death.

Fig. 9) shows binding and killing of T cells from the knee joint of a patient with rheumatoid arthritis using fixed Y. pseudotuberculosis and fixed control y. pseudotuberculosis expressing a truncated Inv.

According to the invention it has been shown that glutaraldehyde-fixed intact bacteria Y. pseudotuberculosis and Y. enterocolitica respectively caused necrotic and apoptotic cell death in T lymphocytes while the same bacterial strain of Y. pseudotuberculosis with a truncated Inv binding site did not. Furthermore, intact bacteria containing a truncated binding site did not provoke cell death even when brought into close contact with T lymphocytes. E. coli, streptoccocci and staphylococci did not kill T cells. It follows that the capacity to kill is specific for Yer (Example 3).

Inv-induced cell death in T lymphocytes is characterized by necrosis and apoptosis. Inv-containing extracts caused cell death in normal T cells and T cell lines and also in T cells from the joints of patients with rheumatoid arthritis Fig. 9. In contrast, an extract of the same strain where Inv carried an insertion in the binding site caused negligible cell death. Noteworthy, a fusion protein containing the Inv binding site for β_1 integrins of lower molecular weight than the whole Inv protein did not cause cell death although it was capable of binding to T cells.

The extracts that were effective in inducing cell death contained a relatively high amount of a 100 kD Inv component while less active extracts contained relatively more of a 70 kD Inv component. Further, the full length Inv protein bound to the cells whereas the truncated Inv did not.

The killing effect of Inv could not be inhibited by inhibitors of the Interleukin 1β converting enzymes that are inhibitors of the main effector pathway for apoptosis induced by a number of triggering factors (Example 2). This further supports the conclusion that Inv induces cell death mainly by necrosis.

Blocking experiments using monoclonal antibodies to β_1-integrin chains, indicate that the β_1-integrin chain which participates in the regulation of integrin affinity is involved in the induction of cell death by Inv.

The possible cell death-inducing effect of Inv has been investigated in several non-lymphoid tumour cells including a lung cancer cell line, a colon adenocarcinoma cell line and a malignant glioma cell line. Extracts of Yer where the 100 kD Inv component dominated were active in inducing cell death in the non-lymphoid tumour cells while extracts where the low mw Inv component was most abundant usually were non-active (Example 4).

Yersinia pseudotuberculosis is an animal disease characterized by necrotic lesions in

liver, spleen and lymph nodes (Volk, W. A. et al. Essentials od Medical Microbiology (Fourth Ed) J. B. Lippincott Co., Philadelphia, 1991). The present finding that a component of Yer species directly can cause necrotic killing of T lymphocytes may explain these in vivo observations.

Virtually all effects of bacteria on the immune system which have been described so far are mediated via the influence of superantigens and lipopolysaccarides which trigger cells of the immune system to proliferate or produce immunoglobulins (Scherer, M. T. et al. Annu. Rev. Cell Biol. 9, 101-128, 1993 and Ulevitch, R. J. et al. Annu. Rev. Immunol. 13, 437-457, 1995). The ability of Inv to kill lymphocytes may play an important role during the course of infection by Yer species. The ability of Inv to kill various neoplastic cells further suggests that Inv or molecules tailored based on Inv characteristics may be applied as a therapeutic tool against cancer.

The use according to the invention of invasin and invasin-like molecules micro-organisms producing them and extracts containing them may thus be an effective tool in treating sicknesses and conditions caused by cells having β_1-integrin.

The invention will now be illustrated by the following non limiting examples.

In the examples the following material and methods have been used.

Antibodies. The monoclonal anti-β_1-integrin (CD29) antibody, clone Lia 1/2, was purchased from Immunotech. The monoclonal anti-CD47 antibody, clone BRIC 126, was purchased from Nordic Biosite AB and anti-CD9 clone MM2/57 and anti-CD63 clone LP9 were purchased from Serotec Ltd. The anti-Inv antibody preparation used in the studies was a mix of the clones 3A2-1, 2F11-12, 9G11-9A, 1B3-3 and 6D7-1 all recognizing the carboxy-terminal region of Inv protein(Leong, J. M. et al. Infect. Immun. 59, 3424-3433, 1991). The rabbit polyclonal antibody used to remove Inv (100

kD fragment) was made by Neosystem Laboratoire using a 15 amino acid (a-a) peptide from the amino-terminal part of the protein (comprising 263-277 a-a).

T-cell lines. Jurkat and Molt-4 cell lines were purchased from American Type Culture Collection. HPB-ALL cell line (β_1-integrin positive) and its parental HAB.7 cell line (β_1-integrin negative) were gifts from Håkan Hedman, Dept. of Cell and Molecular Biology, University of Umeå, Umeå, Sweden. (Present address at La Jolla Cancer Research Center, The Burnham Institute, La Jolla, CA). AF-24, a normal cloned T cell line was obtained from Joost van Neerven ALK, Hoersholm, Denmark. Human peripheral blood T lymphocytes were isolated from buffy coats of healthy donors using a lymphoprep gradient (Nycomed Pharma AS). T cell enrichment was performed by means of magnetic beads coated with antibodies to CD14 and CD19 (Dynabeads, Dynal AS).

Yersinia strains. Yer strains YPIII(pIB1) (Inv positive) and YP100(pIB1) (Inv negative, truncated)(Rosqvist, R. et al., Nature 334, 522-525, 1988) were used in the preparation of semi-purified extracts Y1E and Y2E respectively.

Binding of Inv, apoptotic assay and protease inhibitors. Binding of Inv and fixed bacteria (Y1) to cells was checked using the anti-Inv mAb mix described earlier and cytofluorometry. For the determination of necrotic and apoptotic cell death $1{,}8x10^6$ cells were cultured with 20 ul of Y1E or Y2E (or Y1 fixed bacteria, Y2 fixed bacteria; cells/bacteria ratio 1:20) in a 24 well culture plate at 37 °C, 5% CO_2 incubator 1, 3, 5, 7 or 18 hours. Next day the cells were collected, washed and processed according to protocols for the TUNEL assay using the In Situ Cell Death Detection Kit and/or the Annexin test using the Annexin-V-FLUOS Staining Kit bought from Boehringer Mannheim.

The protease inhibitors Cbz-Val-Ala-Asp(OMe)-fluoromethyl ketone (ZVAD-FMK) and Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (ZDEVD-FMK) were purchased from Enzyme Systems Products.

Example 1. Preparation of invasin extracts.

Yersinia pseudotuberculosis strains YPIII(pIB1) (Inv positive) and YP100(pIB1) (Inv negative, truncated) were used in the preparation of semi-purified extracts Y1E and Y2E respectively. Inv can be obtained from any Yer species. Inv used in the present experiments was obtained from strains of Y. pseudotuberculosis kept in the Department of Clinical Immunology. Briefly, after culturing the strains in LB medium, a standard medium for bacterial culture, at 26 °C the pellets were resuspended in Tris-HCl pH 6,7 and an extraction of outer membrane proteins was carried out using 6M urea. After centrifugation at 5000 g for 15 minutes 4 °C the supernatant with soluble proteins was collected and extensively dialyzed against distilled water and lyophilyzed. The concentrated material was dissolved in a small volume and then separated on a S-300 gel filtration chromatography column (Pharmacia). Fractions containing proteins of high molecular weight were pooled and applied in phenyl sepharose (high performance) hydrophobic interaction chromatography (Pharmacia). Fractions containing only a few bands with an approximate mw of 100 kD were checked with respect to the presence of Inv and binding to cells by immunoblots and cytometry.

Example 2. Development of cell death in T lymphocytes exposed to fixed Yersinia pseudotuberculosis and Yersinia enterocolitica.

Development of necrotic and apoptotic cell death in T lymphocytes exposed to fixed Yersinia pseudotuberculosis(Fig. 1) and Yersinia enterocolitica (Fig. 2) was investigated. Jurkat T lymphocytes were cultured in RPMI, a standard cell culture medium available from different manufacturers, in the presence of 20 glutaraldehyde-

fixed bacteria per cell. Necrotic and apoptotic cell death was determined after 1, 3 and 5 hours using the annexin test according to the instructions from the manufacturer (Boehringer Mannheim).

The results in Fig. 1 and 2 show that glutaraldehyde-fixed intact bacteria Y. pseudotuberculosis and Y. enterocolitica respectively caused necrotic and apoptotic cell death in T lymphocytes.

The killing effect of Inv could not be inhibited by inhibitors of the Interleukin 1β converting enzyme (ICE) and CCP32 such as Cbz-Val-Ala-Asp(OMe)-fluoromethyl ketone (ZVAD-FMK) and Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (ZDEVD-FMK) which are inhibitors of the main effector pathway for apoptosis induced by a number of triggering factors (Rowan, S. et al., Leukemia 11, 457-465, 1997)(Table I and II). This further supports the conclusion that Inv induces cell death mainly by necrosis.

Table I. The influence of interleukin-1β converting enzyme (ICE) inhibitor ZVAD-FMK on the cell death inducing effect of Y1 in Jurkat cell line.

Cell line	Y1 fixed	ZVAD+ Y1 fixed	ZVAD	FasL	ZVAD+ FasL
Jurkat	50,65[(a)]	46,99	0,01	84,87	2,93

(a) Values represent percentage dead cells.

Table II. The influence of cysteine protease (CCP-32) inhibitor ZDEVD-FMK on the cell death inducing effect of Y1 in Jurkat cell line.

Cell line	Y1 fixed	ZDEVD+ Y1 fixed	ZDEVD	FasL	ZDEVD+ FasL
Jurkat	19,44[(a)]	21,57	0,26	31,76	7,82

(a) Values represent percentage dead cells.

Example 3. Cell death caused by Y1 and Y2 (truncated) extracts from Yersinia pseudotuberculosis and Maltose Binding Protein (MBP) fusion proteins Inv-740 and Inv-497.

T lymphocytes, Jurkat and AF-24 cell line, were cultured in RPMI media in the presence of extracts obtained as in example 1 from Yersinia pseudotuberculosis Y1 and Y2 (Y1E and Y2E, respectively) for 14 hours. Cell death was determined by the Annexin test as in Example 1.

The results in Fig.3 show that a semi-purified extract of Yersinia pseudotuberculosis containing Inv caused cell death in T lymphocytes characterized by necrosis and apoptosis. Inv caused some apoptotic cell death as showed by the Annexin test but this comprised about 25% of the total number of dead cells. The MBP fusion protein Inv-740 caused apoptotic cell death in AF24 and Jurkat T cells. Furthermore, the intact bacteria Y2, containing a truncated binding site, did not provoke cell death even when brought into close contact with T lymphocytes.

Example 4. Comparison of the capacity of three different extracts from Y. pseudotuberculosis with different molecular weight to induce killing of Molt-4 T lymphocytes.

The capacity of three different extracts from Y. pseudotuberculosis with different molecular weight to induce killing of Molt-4 cells was investigated. The extracts were obtained as described in example 1 and the molecular weight thereof are shown in Fig.4. The extract Y1A was the most effective in killing Molt-4 cells. Y1A shows only traces of the lower Inv fragment (64 kD) and an upper fragment (103 kD) predominates as demonstrated by western blotting using an anti-Inv mAb mix. Lanes 1-2, MBP-Inv (fusion protein) positive control, non-reduced(*) and reduced(**) respectively. Lanes 3-4, Y1 13/2 extract; 5-6, Y1A extract; 7-8, Y1C extract. Standard markers on the left side. The extracts were used in cell cultures and percentage cell death was as follows: Y1 13/2= 13,18%; Y1A=67,36% and Y1C=8,96%.

The extracts that were effective in inducing cell death contained a relatively high amount of a 100 kD Inv component while less active extracts contained relatively more of a 70 kD Inv component Fig. 4. A possible explanation for this may be that the "low molecular weight" (mw) Inv blocks binding of the 100 kD protein. The low mw fragment is probably generated from the high mw component by proteolytic degradation.

Example 5. Binding of Y1 and Y2 Invasin from Yersinia pseudotuberculosis to T lymphocytes. (Jurkat and AF-24 cell lines.)

The Jurkat and AF-24 cell lines were cultured in the presence of Y1 and Y2 extracts obtained as in example 1 for 14 hours. Binding of Inv was detected using a mix of monoclonal antibodies to Inv and FACS analysis.

Fig. 5 shows that the full length Inv protein bound to the cells whereas the truncated Inv did not.

Example 6. Cell death-inducing effect on Jurkat T lymphocytes of an extract of

Yersinia pseudotuberculosis (Y1E) before and after removal of Inv (100 kD).

Cell death-inducing effect on Jurkat cells of an extract of Yersinia(Y1E) before and after removal of Inv (100 kD) was studied. Removal of Inv was carried out by passing the extract Y1E through a rabbit anti-Inv-coupled Sepharose 4B column according to the instructions of the manufacturer. After collection of the passed fractions (Y1E-Inv) they were used in comparative killing experiments with the nonabsorbed extracts. Cell death was determined after 14 hours as previously described.

From Fig. 6 it is evident that removal of the 100 kD fragment from the Yer extract reduced the cell death-inducing effect markedly.

Example 7. influence of monoclonal antibodies to cell surface antigens on Inv-induced cell death.

The influence of monoclonal antibodies to cell surface antigens on Inv-induced cell death in Jurkat cells was studied. This was done in order to elucidate via which surface receptors Inv mediated the killing effect in T lymphocytes blocking antibody experiments were performed using monoclonal antibodies to β_1-integrin chains, CD47, CD9, and CD63 which were associated to integrin function. The cells were cultured on RPMI medium for 14 to 16 hours in the presence of Y1E and Y2E with and without monoclonal antibodies to different cell surface antigens. Cell death was determined at the end of the culture period.

Anti-CD29 consistently exhibited a 50% blocking of the cell death-inducing effect of Inv (Fig. 7). In contrast, antibodies to α_4 and α_5 subunits did not block killing. The results of the antibody blocking experiments indicate that the β_1-integrin chain which participates in the regulation of integrin affinity is involved in the induction of cell death by Inv.

Example 8. Reduction of Inv-induced cell death in a β_1-integrin negative cell line (HAB.7) compared with its parental line (HPB-ALL).

Further support for the conclusion that the β_1-integrin subunit participated in the induction of cell death was obtained when the killing effect of Inv was studied in a β_1-integrin negative T lymphocyte cell line HAB.7 Cells of (HAB.7) and its parental line HPB-ALL respectively were cultured with fixed Yersinia pseudotuberculosis for 16 hours before determination of cell death.

From Fig. 8 it is evident that the β_1-integrin negative T lymphocyte cell line HAB.7 exhibited a 50% reduction of killing compared with the parental cell line. A reasonable explanation why the reduction of killing was not 100% may be that interaction via some other lymphocyte surface component contributed to the Inv induced cell death.

Example 9. Cell death in non-lymphoid tumour cells .

The possible cell death-inducing effect of Inv has been investigated in a number of other cell types in addition to T lymphocytes. Yersinia strains (Y1, Inv+ and Y2 Inv-), obtained as described in Example 1, were fixed in glutaraldehyde 0,25% for 3 minutes at 4 °C and used to study binding as described in Example 5 and killing effects as described in Example 3. The results which are summarized in Table III demonstrate that Inv caused necrotic/apoptotic cell death in several non-lymphoid tumour cells including a lung cancer cell line, a colon adenocarcinoma cell line and a malignant glioma cell line. Extracts of Yer where the 100 kD Inv component dominated were active in inducing cell death in the non-lymphoid tumour cells while extracts where the low mw Inv component was most abundant usually were non-active.

Table III. Cell death inducing effect of Inv in different tumour cell lines.

Malignant cell lines	% Cells binding Inv		% Cell death	
	Y1fixed	Y2fixed	Y1fixed	Y2fixed
LS 174T[(a)]	28,71	6,78	21,25	6,81
LC Wart[(b)]	76,27	28,03	70,33	8,79
MG 251[(c)]	75,80	0,68	48,77	0,70

Y1 bacteria show high binding and killing while the truncated negative control Y2 shows relatively low binding and killing.

(a) Adenocarcinoma cell line.

(b) Lung cancer cell line.

(c) Malignant glioma cell line.

Example 10. Y. pseudotuberculosis (fixed bacteria) induced cell death in rheumatoid joint T cells.

Fixed Y. pseudotuberculosis and/or Inv-containing extracts from this bacteria caused cell death in normal T cells and T cell lines and also in T cells from the joints of patients with rheumatoid arthritis. In contrast, a Y. pseudotuberculosis strain with a truncated Inv binding site or an extract from this strain caused negligible cell death.

Fig. 9 shows binding and killing of fixed Y. pseudotuberculosis (Y1 fixed) compared with Y. pseudotuberculosis control carrying a truncated Inv (Y2 fixed) and their effects on rheumatoid arthritis T lymphocytes.

Example 11. Development of antibodies to β_1-integrins with capacity to kill cells.

The monoclonal antibodies of the present invention are prepared using classical cloning and fusion techniques. Mice of the appropriate strain are immunized with purified β_1-integrins. Purified $\alpha_5\beta_1$-integrin (1-50 ug), commercially available (Chemicon AB), is used as immunogen for the mouse immunization in a standard protocol for monoclonal

antibody production (Ref. 1. Oi, V. T. and Herzenberg, L. A. 1980. Immunoglobulin-producing hybrid cell lines. In Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi. eds. pp 351-372. W. H. Freeman, New York. 2. Monoclonal antibodies production. Current Protocols in Immunology. Vol. I. Ed. J. E. Coligan, et al. Natl. Inst. Health. John Wiley and Sons, Inc. 1998, New York.). After the mouse has been boosted three times the spleen is removed and splenocytes are extracted and fused with myeloma cells using the well-known processes of Kohler and Milstein (Nature 256:495-497, 1975). The resulting hybrid cells are then cloned in the conventional manner, e.g., using limiting dilution, and the resulting clones are screened.

ELISA microtiter plates are set up with lymphoid cells as targets for screening of hundreds of clones. Supernatants with killing activity permeabilize the target cell membrane and the membrane injury is assessed by lactate dehydrogenase or chromium 51 release. Permeable necrotic/apoptotic cells also incorporate a fluorescent dye in their nuclei that is detected by a reader system (ELISA, FACS Cytometry, etc) identifying a supernatant with killing activity and so the specific clone(s).

Alternative techniques may also be utilized to construct monoclonal antibodies and/or portion of antibodies using antibodies expression libraries and recombinant DNA techniques respectively for which there are numerous references. (see William D. Huse et al., "Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda", Science 246:1275-1281, December 1989; see also Michelle Alting-Mees et al., "Monoclonal antibody expression library: a rapid alternative to hybridomas", Strategies in Molecular Biology 3:1-9, January 1990.)

Claims

1. Use as an active substance of molecules binding to β_1-integrin and with the capacity to kill cells having β_1-integrin for production of pharmaceuticals for treatment of conditions such as cancer and conditions dependent on T lymphocytes, and fibrotic conditions.

2. Use according to claim 1 characterized in that the molecules binding to β_1-integrins are chosen from invasin, subfragments, variants, and peptides thereof, or other molecules with invasin like effect and microorganisms producing such substances or antibodies that kill tumor cells and/or T-lymphocytes and other cells having β_1-integrin for production of pharmaceuticals for treatment of conditions dependent on cells having β_1-integrin such as cancer and conditions dependent on T- lymphocytes and fibrotic conditions.

3. Use according to claim 1, characterized in that live or fixed microorganisms or extracts from live or fixed microorganisms belonging to the Yersinia group are used, especially Yersinia pseudotuberculosis, Yersinia pestis or Yersinia enterocolitica.

4. Use according to claim 1 or 2, characterized in that the substance, subfragments, variants, and peptides thereof or microorganism or extract from microorganism is coupled to a target seeking monoclonal or polyclonal antibody, Fab or Fc portion of a polyclonal antibody or monoclonal antibody or a hybrid antibody or other proteins.

5. Use according to claim 1, characterized in that the molecule capable of binding to β_1-integrin is a monoclonal antibody or hybrid antibody.

6. Use according to anyone of claim 1-4, characterized in that the conditions dependent on (caused by) T-lymphocytes are autoimmune diseases e.g. rheumatoid arthritis, rejection of foreign transplants and fibrotic conditions.

7. Use according to anyone of claim 1, 3 and 5, characterized in that the invasin gene or sequence of the invasin gene is used as part of specially designed gene therapy for delivery in specific locations as brain, joints, liver, etc, as well as in other therapeutic method(s).

8. Pharmaceutical composition for use according to anyone of claims 1-6, characterized in that it comprises a pharmaceutical active amount of invasin, subfragments, variants, and peptides thereof, and microorganisms producing such substances, antibodies with invasin like effects, the invasin gene or sequence of the invasin gene to kill tumour cells and/or T-lymphocytes and other cells having β_1-integrin possibly together with additives or excipients.

9. A method for killing of T lymphocytes comprising exposing the lymphocytes to an effective amount of live or fixed Yersinia pseudotuberculosis, Yersinia pestis or Yersinia enterocolitica, an extract of live or fixed Yersinia pseudo/pestis/entero or to Yersinia pseudo/pestis/enterocolitica invasin.

10. A method for killing of tumour cells comprising exposing the tumour cells to an effective amount of live or fixed Yersinia pseudotuberculosis, Yersinia pestis or Yersinia enterocolitica, an extract of live or fixed Yersinia pseudo/pestis/entero or to Yersinia pseudo/pestis/enterocolitica invasin.

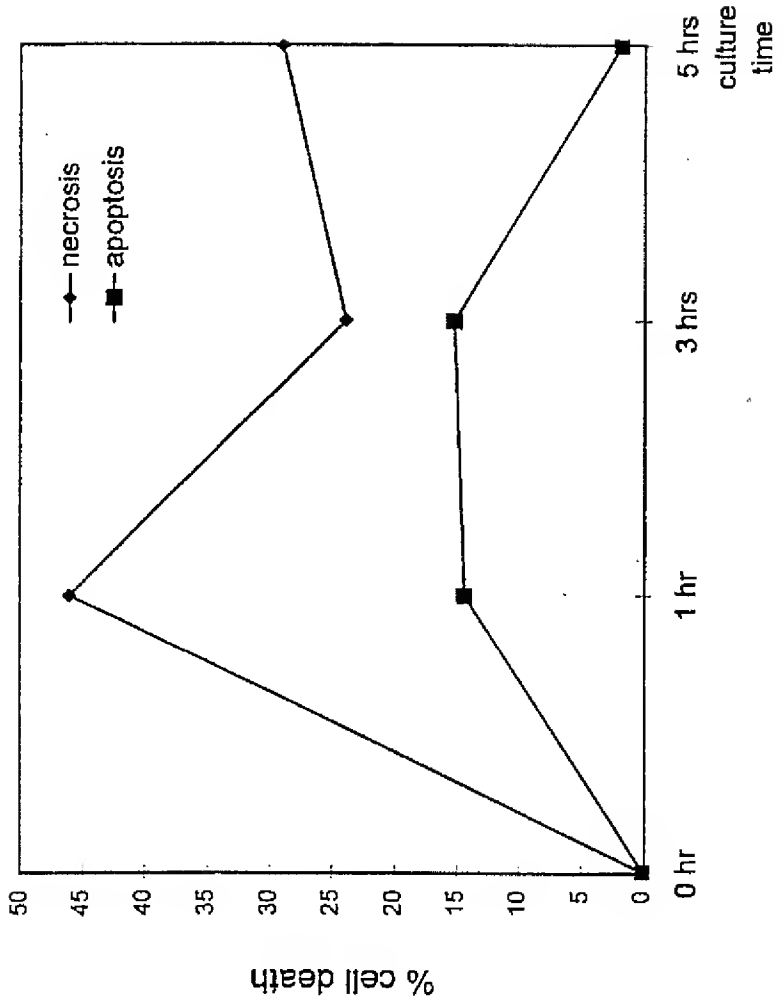

necrosis
apoptosis
% cell death
50
45
40
35
30
25
20
15
10
5
0
0 hr
1 hr
3 hrs
5 hrs
culture time

Fig. 1

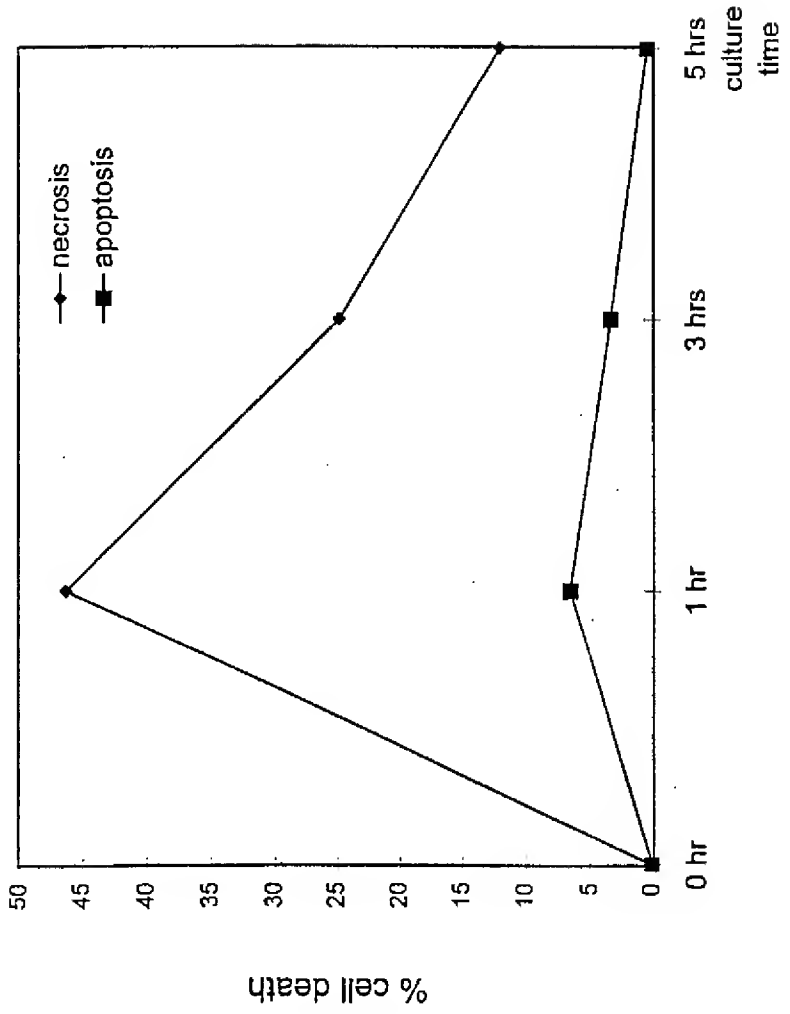

necrosis
apoptosis
% cell death
0
5
10
15
20
25
30
35
40
45
50
0 hr
1 hr
3 hrs
5 hrs
culture time

Fig. 2

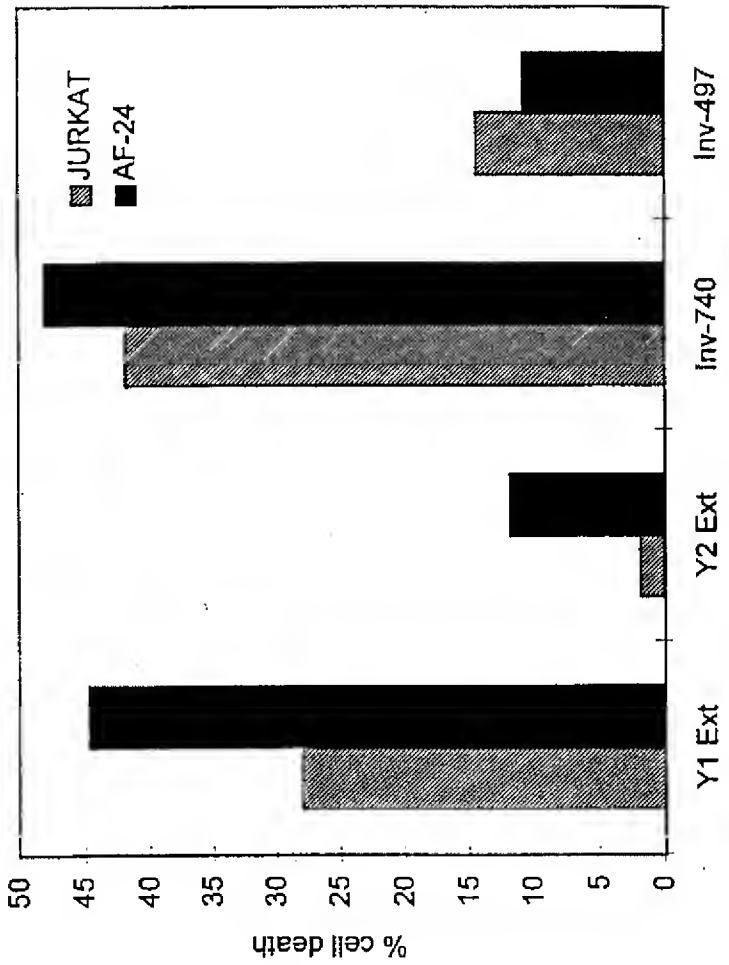
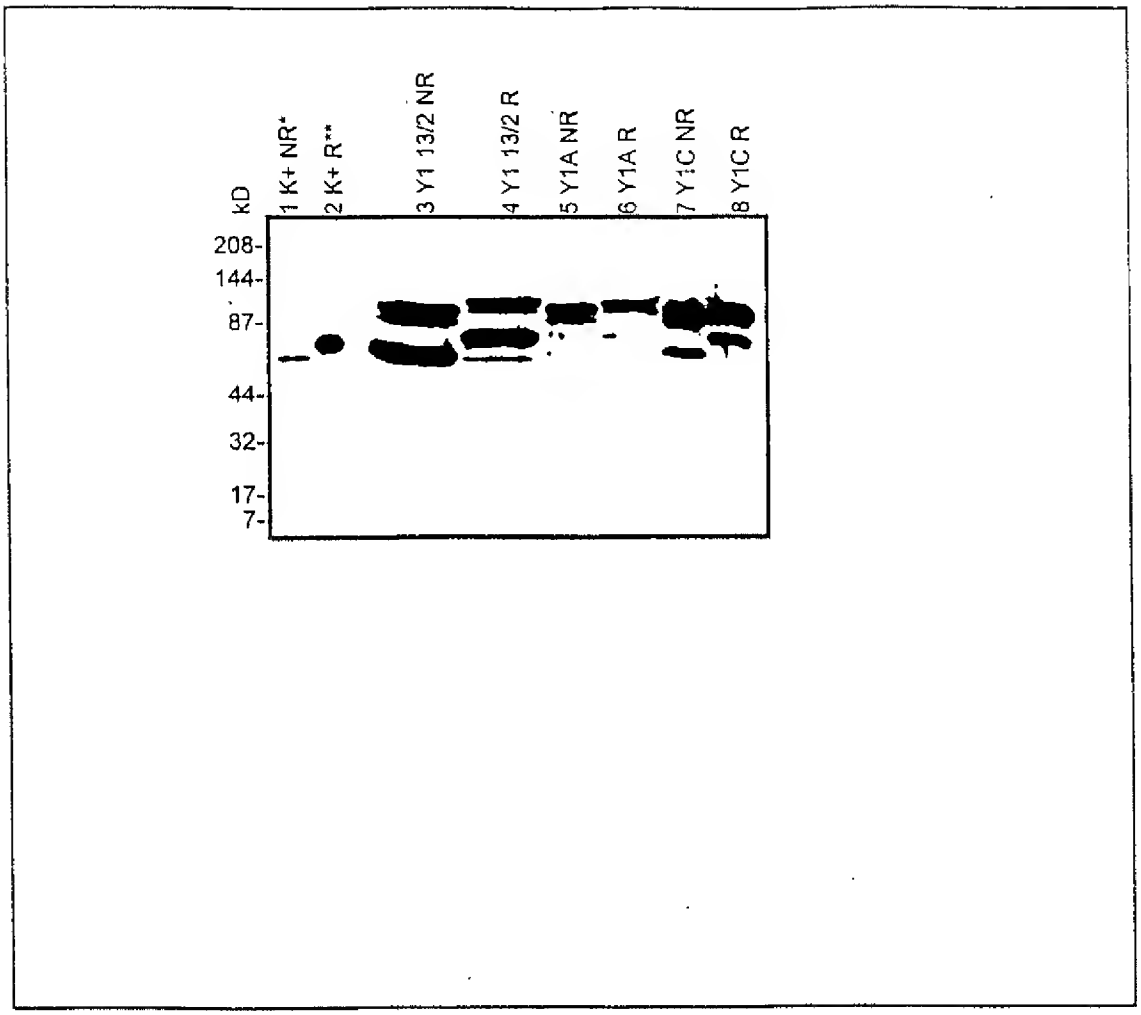

JURKAT
AF-24
Inv-497
Inv-740
Y2 Ext
Y1 Ext
% cell death
50
45
40
35
30
25
20
15
10
5
0

Fig. 3

Fig. 4


kD
1 K+ NR*
2 K+ R**
3 Y1 13/2 NR
4 Y1 13/2 R
5 Y1A NR
6 Y1A R
7 Y1C NR
8 Y1C R
208-
144-
87-
44-
32-
17-
7-

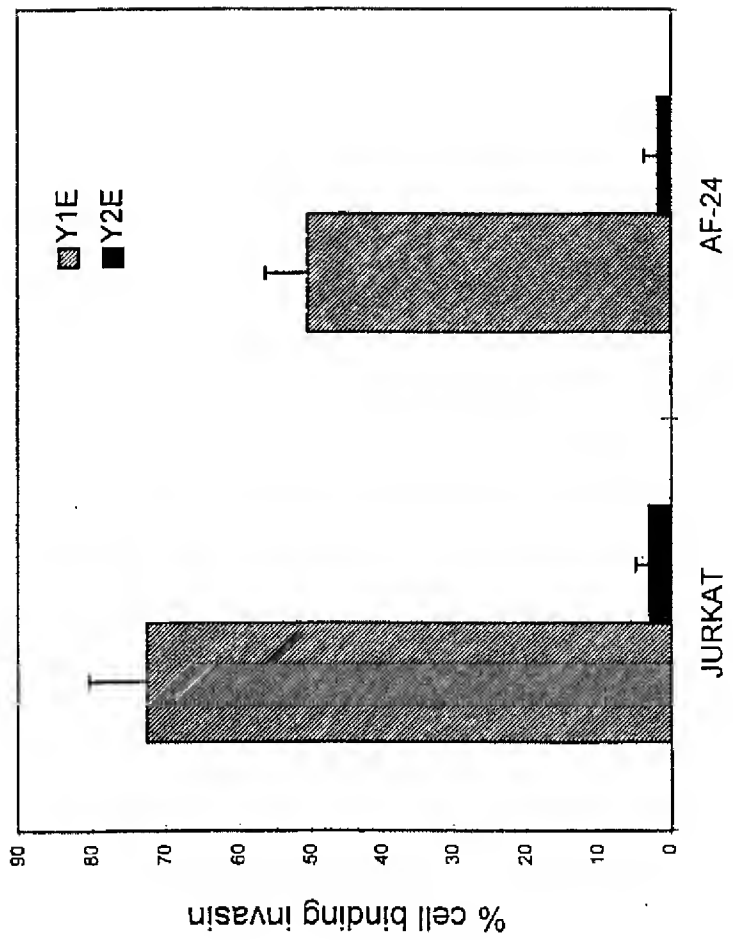

Y1E
Y2E
AF-24
JURKAT
% cell binding invasin
0
10
20
30
40
50
60
70
80
90

Fig. 5

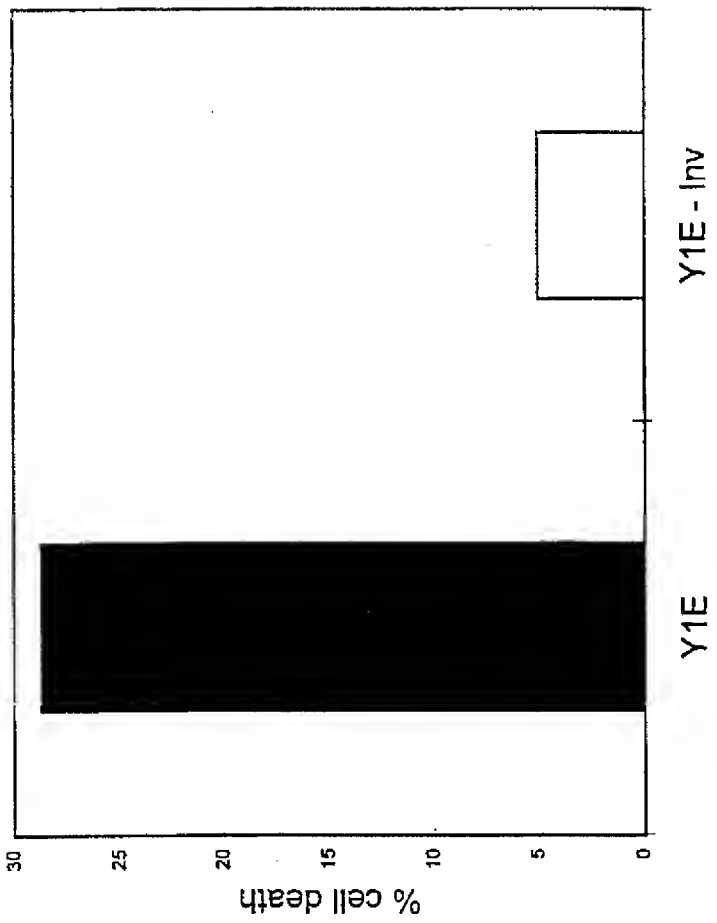


Fig. 6

Fig. 7

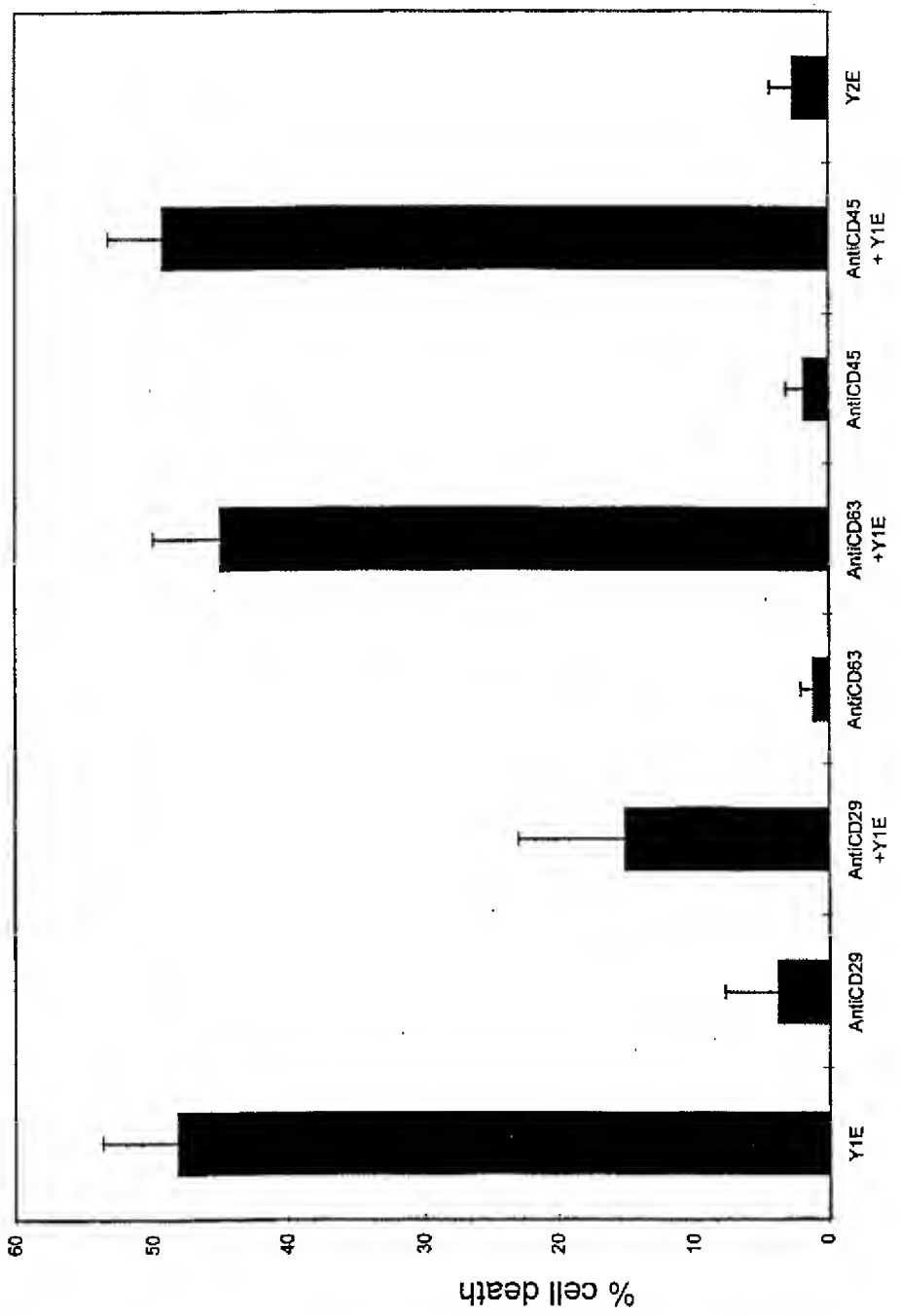

% cell death
0
10
20
30
40
50
60
Y1E
AntiCD29
AntiCD29 +Y1E
AntiCD63
AntiCD63 +Y1E
AntiCD45
AntiCD45 + Y1E
Y2E

Fig. 8

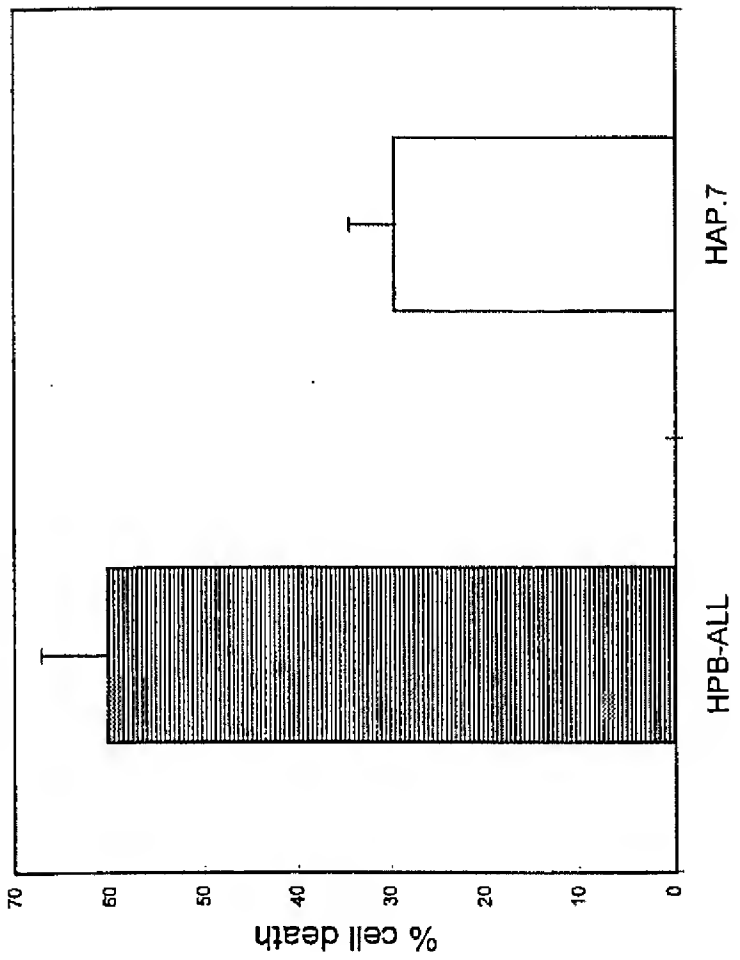
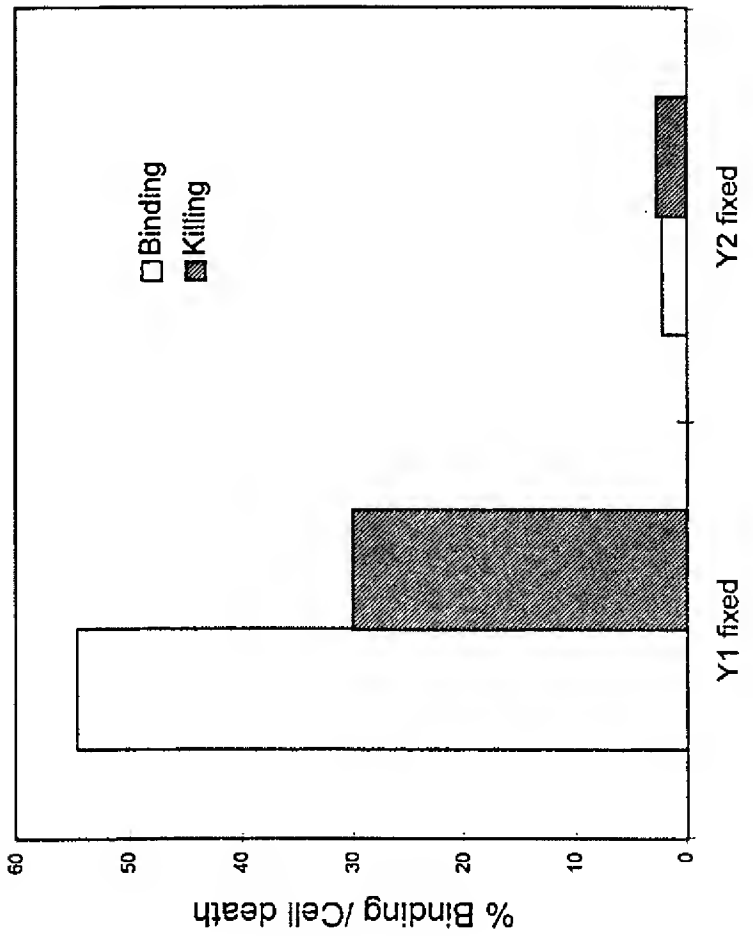


Fig. 9


Binding
Killing
Y2 fixed
Y1 fixed
% Binding /Cell death
60
50
40
30
20
10
0